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# Isolation and Characterization of an Avian Adenovirus-Associated Virus

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An 18- to 20-nm virus particle was isolated from the Olson strain of quail bronchitis, an avian adenovirus. On density gradient separation the small virions were primarily found at densities of 1.39 and 1.42 g/cm$^3$. The majority of the infectious particles were at the heavier density. The virus had a hexagonal outline and contained single-stranded deoxyribonucleic acid. It was resistant to heating at 56 C for more than an hour and was not inactivated by treatment with chloroform or low pH. Purified virus did not agglutinate erythrocytes of various avian and mammalian species. Replication of the small particles occurred either in chicken embryos or in cultures of embryo kidney cells coinfected with an adenovirus helper. Antigenically the virus was distinct from the adeno-associated viruses types 1, 2, 3, and 4. The virus is the avian equivalent of the adeno-associated viruses of primates and lower animals.

The adeno-associated viruses are defective and have been found in association with the adenoviruses of man (M.D. Hoggan, Fed. Proc. **24**:248, 1965), monkeys (4), cattle (13), turkeys (22), and dogs (25). This report describes the biophysical, growth, and antigenic properties of an avian adeno-associated virus (AAAV) which was isolated during concentration and purification of the Olson strain of quil bronchitis virus (20).

## MATERIALS AND METHODS

**Virus.** AAAV was propagated in SPAFAS embryonated eggs (Norwick, Conn.) coinfected with either the Olson isolate of quail bronchitis or chicken-embryo-lethal-orphan (CELO) virus (27). The viruses were inoculated either simultaneously, or the adenovirus exposure preceded that of AAAV by one to several hours. Inocula were introduced via the chorioallantoic sac. Embryos were observed daily for retarded growth or death. When approximately 20% of the embryos succumbed to the dual infection all of the eggs were chilled, and the infected allantoamniotic fluids (AAF) were harvested.

The adeno-associated viruses (AAV) types 1, 2, and 3 were obtained from M. D. Hoggan; type 4 was obtained from H. D. Mayor. AAV 1, 2, and 3 were also propagated in chicken embryos coinfected with CELO virus.

**Assay for infectivity, complement fixing, and immunofluorescent antigens.** The infectivity of AAAV was assayed in 10-day chicken embryos. Sets of embryos were dually infected with various decimal dilutions of AAAV and $10^5$ plaque-forming units of CELO virus. After incubation for 4 days, the AAF of each egg was harvested and screened for AAAV specific antigens by complement fixation (CF). The mean embryo infective dose ($EID_{50}$) titer of virus was calculated.

The CF test has been described (14). Antibody titers were determined by checkerboard titrations against homologous and control antigens. Antigen titers were determined versus 4 to 8 U of antibody.

An indirect immunofluorescence technique was used (8). Fluorescein-labeled goat anti-rabbit globulin was obtained from Hyland Laboratories, Costa Mesa, Calif. Appropriate dilutions of antiserum and conjugate were determined prior to use. All observations employed a 4-mm BG 38 heat filter, a 2-mm BG 12 exciter filter, and a blue-excluding barrier filter.

**Purification of AAAV.** The technique was adapted from that of Laver et al. (12). Infective AAF was clarified by centrifugation at 8,000 × *g* for 10 min. Using a burette, approximately 25 ml of the supernatant fluid was layered on a 6-ml cushion of 42% CsCl in a SW 25 (Spinco) rotor tube. Centrifugation was at 50,000 × *g* for 1 h with the brake off. The fluid above the bands and cushion was removed, and additional clarified AAF was added. After two or three cycles of refilling and centrifugation at 50,000 × *g*, the opaque bands containing the virus particles were harvested through the side of the tube by using a syringe. The virus suspensions from all tubes were pooled, and crystalline CsCl was added to give a specific gravity of 1.38 g/cm$^3$. This fluid was then used to fill SW 39 (Spinco) rotor tubes. Centrifugation was at 100,000 × *g* for 20 to 24 h.

The bands that formed at densities of approximately 1.39 and 1.42 g/cm$^3$ were harvested through

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the side of the tube and placed in a SW 39 rotor tube. CsCl was added to the suspension until the average density was again 1.38 g/cm³. Centrifugation was at 100,000 × *g* for 40 to 44 h. The bands were removed through the side of the tube or via a needle inserted in the base of the tube. For some procedures the banded virus suspensions were diluted 1:10 with Hanks balanced salt solution (HBSS) and filtered by using a 50-nm membrane filter (Millipore Corp.).

**Specific gravity in CsCl.** After the second CsCl density gradient centrifugation (40–44 h at 100,000 × *g*), 12-drop fractions were collected via a needle inserted in the base of the tube. After determining the specific gravity of each fraction by using an ABBE refractometer (Bausch and Lomb), HBSS was added to make a 1:10 dilution. These samples were stored at −20 C until assayed for AAAV.

**Electron microscopy.** Negative staining was carried out as follows. For morphological studies one drop of virus suspension or virus-antibody mixture was placed on small squares of 2% Noble agar. After drying, collodium pseudo-replicates were made and stained by floating on 1% phosphotungstic acid at pH 7. The membranes were picked up on grids and examined in a Siemens Elmiskop 1A electron microscope.

Thin sections were made as follows. Chorioallantoic membranes (CAM) infected with AAAV alone or with AAAV and CELO were fixed in 3% glutaraldehyde, postfixed with $OsO_4$, embedded in Araldite, stained with uranyl acetate and lead citrate, sectioned, and examined.

**Acridine orange staining.** Suspensions of purified AAAV, of CELO virus purified by the technique of Anderson et al. (2), and of a hamster type-C virus (D-9; reference 24) purified by sucrose density gradient centrifugation were stained with acridine orange according to the procedure of Mayor (16). Suspensions of each virus were also treated with either ribonuclease (RNase) or deoxyribonuclease (DNase) prior to staining with acridine orange.

**Susceptibility to heat, chloroform, and pH 3.** One-milliliter samples of density gradient purified AAAV, diluted in HBSS to contain $10^7$ $EID_{50}$/ml, were heated at 56 C for 30, 60, or 90 min.

Similar virus suspensions were treated with chloroform by the method of Feldman and Wang (6) or held at pH 3 in citrate-phosphate borate buffer (26) at room temperature (7). After treatment the suspensions were assayed for AAAV infectivity.

**Hemagglutination.** Erythrocytes were obtained from the following species: human (type 0), guinea pig, rat, dog, hamster, chicken (0.5-day and adult), horse, goose, monkey, rabbit, and pig. After washing the cells with phosphate-buffered saline, 1% suspensions were made in phosphate-buffered saline at pH 7.2. These cellular suspensions were used in a microtest (2) at either 4 or 37 C to test the agglutinability of density gradient purified AAAV.

**Growth in chicken embryos.** Eleven-day chicken embryos (SPAFAS) were simultaneously infected with AAAV and CELO virus. The inocula were deposited in the chorioallantoic sac with a tuberculin syringe. Embryos were observed daily for retarded growth or death. At various times after infection, two embryos were removed from the incubator and their AAF and CAM were harvested. Two milliliters of the AAF harvested from each of the embryos were pooled and stored at −20 C until assayed for CF antigen. One portion of the CAM from each embryo was placed in 3% glutaraldehyde and another in 10% neutralized Formalin. The tissues fixed in Formalin were processed and stained with hematoxylin and eosin for histological examination.

**Growth in chicken embryo kidney cells.** Chicken embryo kidney (CEK) cells were prepared (3) and seeded in Leighton tubes with cover slips. When confluent, the cell sheets were simultaneously infected with AAAV and CELO virus. At 10, 20, 24, 48, and 96 h after exposure, 4 tubes were removed from incubation. The supernatant fluids were pooled at each harvest and stored at −20 C until assayed for CF antigens. Cover slips were removed and processed for immunofluorescence studies.

**Preparation of antisera.** AAV 1 and AAAV were purified from infected AAF by two density gradient centrifugations. Virus bands were diluted 1:10 with HBSS and filtered through a 50-nm membrane filter (Millipore Corp.). Three milliliters of the filtered suspension were homogenized with Freund complete adjuvant and used to inoculate two rabbits intramuscularly. Seven to ten days after the first exposure, the rabbits were given one milliliter of the filtered virus suspension intravenously. The rabbits were bled two weeks after the intravenous injection. The serum was stored at −20 C. Antiserum to AAV types 2, 3, and 4 were obtained from H. D. Mayor.

**Viral aggregation.** Suspensions of each virus, either density gradient purified or tissue culture propagated, were mixed with 1:2 or 1:10 dilutions of antiserum. The serum-virus mixtures were incubated for 1 h at 37 C and processed for viewing with the electron microscope.

## RESULTS

**Density gradient centrifugation.** In the purification of quail bronchitis virus by equilibrium centrifugation in CsCl, a diffuse band appeared below that of the adenovirus. By electron microscopy, AAAV particles were seen both in the diffuse band and in the band containing the adenovirus. Both bands were harvested, pooled, and centrifuged in CsCl at 100,000 × *g* for 40 to 44 h. Three light-scattering bands formed at densities of 1.34, 1.38, and 1.42 g/cm³ (Fig. 1). The upper band contained adenovirus virions as well as a mixture of complete and incomplete small particles. AAAV was found primarily in the heavier bands, and the purest preparation was at the density of 1.42 g/cm³.

On fractionation of the gradient, 2 peaks of AAAV-specific activity were found (Fig. 2). The larger amount of CF antigenic material was present at a density of 1.39 g/cm³. The smaller peak at a density of 1.42 g/cm³ contained a majority of infectious virus.

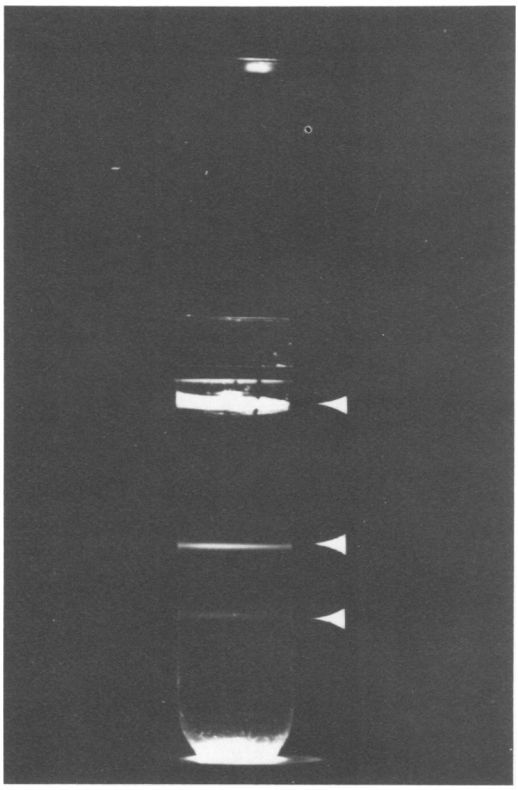

FIG. 1. *Bands of virus concentration, after density gradient separation in CsCl. Centrifugation was at 100,000 × g for 40 h. The upper band (arrow) had a density of 1.34 g/cm³, the middle (arrow) had a density of 1.39 g/cm³ and the bottom (arrow) had a density of 1.42 g/cm³. AAAV was found in all bands, but in higher concentration in the lower two. Densities were calculated from refractive indices.*

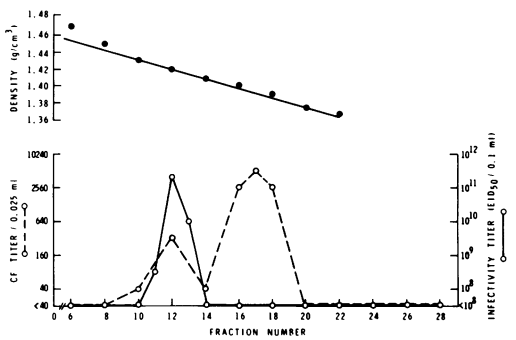


FIG. 2. *Complement fixing and infectivity titers of AAAV antigens in fractions of a CsCl density gradient. Centrifugation was at 100,000 × g for 40 h. Twelve-drop fractions were collected through the bottom of the tube. Densities were calculated from refractive indices.*

**Morphology.** On electron microscopic examination of negatively stained preparations, the virion had a hexagonal outline, suggestive of icosahedral symmetry; however, the capsomeric structure was not resolved sufficiently to distinguish symmetry (Fig. 3). The size of the particle varied from 18 to 21 nm in all populations, regardless of densities. Incomplete particles were found at both densities, but were more frequent at 1.39 g/cm³.

**Nucleic acid.** Acridine orange staining of a purified AAAV suspension indicated that the particle had a nucleic acid core of single-stranded deoxyribonucleic acid (DNA; Table 1). The flame-red staining was not seen when the preparation was first treated with DNase. Prior treatment with RNase did not alter the staining with acridine orange. The single-stranded ribonucleic acid virus (D-9) and the double-stranded DNA virus (CELO) stained in the proper manner, and the staining was altered by RNase and DNase, respectively.

**Susceptibility to heat, chloroform, and pH 3.** The susceptibility of the virus to heat, chloroform, and pH 3 is shown in Table 2. AAAV was resistant to heating at 56 C for more than 1 h and was not inactivated by treatment with chloroform or low pH.

**Hemagglutination.** Density gradient purified suspensions of AAAV did not agglutinate erythrocytes of nine mammalian or two avian species at either 4 or 37 C.

**Replication in chicken embryos.** When purified AAAV was diluted $10^{-3}$ and inoculated into 11-day embryos coinfected with $10^5$ plaque-forming units of CELO virus, replication could be detected by the CF test (Fig. 4). CF antigens produced by CELO virus were first found at 36 h postinfection. The highest titers were obtained at 72 h. AAAV-specific CF antigens were detected in samples at 72, 96, and 120 h after infection. No induction of AAAV-specific antigens was obtained unless helper adenovirus was present.

On histological examination of the CAM by light microscopy, intranuclear inclusions typical of CELO virus were found in a few endodermal cells at 24 h postinfection. Inclusions were noted only in embryos infected with CELO virus or CELO virus and AAAV. The number of inclusions increased after 24 h with maximal numbers by 48 to 72 h.

On electron microscopic examination of CAM of embryos coinfected with CELO and AAAV, CELO particles were found within the nucleus at 18 h. AAAV was easily detected in nuclei at 48 h after infection (Fig. 5). Although it was uncommon, small numbers of AAAV were

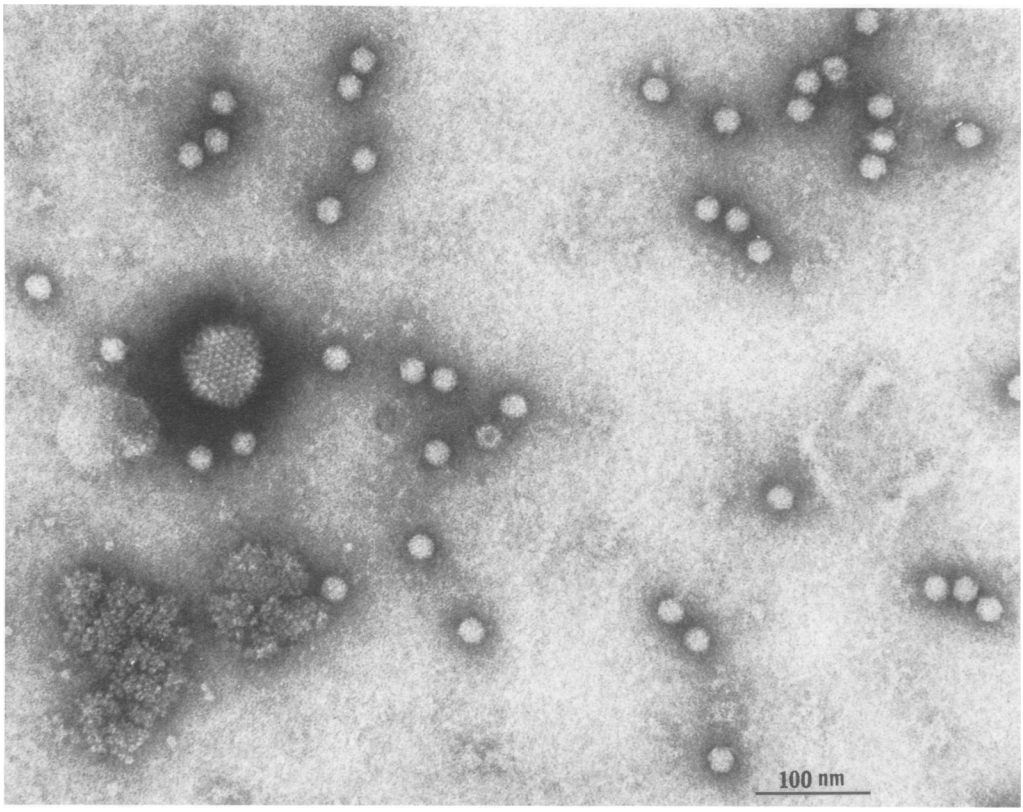


FIG. 3. *Negative stain (1% phosphotungstic acid) of virus from a CsCl density gradient showing AAAV particles and a helper adenovirus particle. Free adenovirus capsomeres appear in the lower left corner.*

TABLE 1. *Staining of AAAV with acridine orange*

| Virus | Pretreatment | | | |
|---|---|---|---|---|
| | None | RNase | DNase | Nucleic acid[a] |
| AAAV[b] | Flame red | Flame red | Color-less | SS-DNA |
| CELO | Green | Green | Color-less | DS-DNA |
| Hamster type C ($D_9$) | Flame red | Color-less | Flame red | SS-RNA |

[a] Abbreviations: SS, single stranded; DS, double stranded.

[b] Viruses purified by density gradient separations.

TABLE 2. *Susceptibility of AAAV to heat, chloroform, and low pH*

| Treatment | $Log_{10}EID_{50}$[a] |
|---|---|
| Untreated[b] | 5.8 |
| Heat, 56 C for 30 min | 6.0 |
| 60 min | 5.8 |
| 90 min | 5.0 |
| Chloroform | 6.8 |
| pH 3 | 5.7 |

[a] Assayed by induction of AAAV specific CF antigen in AAF of embryos coinfected with $10^5$ PFU of CELO virus.

[b] Density gradient purified AAAV was diluted to contain $10^6$ $EID_{50}$/0.1 ml.

found in nuclei containing large numbers of CELO virus. The satellite virus was not found in CAM cells singly infected with the adenovirus.

**Replication of AAAV in chicken embryo kidney cells.** AAAV replicated in CEK cells coinfected with CELO virus. By indirect immunofluorescence, CELO and AAAV viral antigen was detected in a few cells at 24 h postinfection (Table 3). The number of nuclei producing both viral antigens increased by 48 h, at which time the cytopathic effect due to the adenovirus was becoming extensive. CELO-specific CF antigens first appeared in supernatant fluids at 48 h. AAAV-specific CF antigens were not seen until the next sampling at 96 h postinfection.

**Viral aggregation.** The relationship of AAAV to the primate adeno-associated viruses

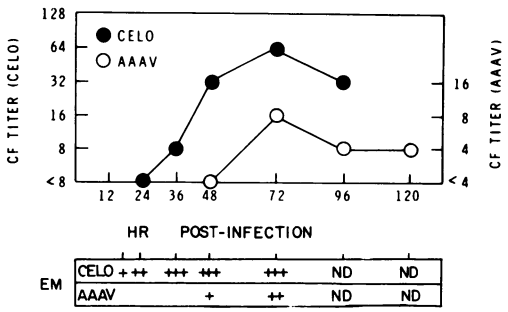


FIG. 4. *Replication of AAAV and CELO virus helper in chicken embryos. Embryos were simultaneously infected with AAAV and CELO virus. At intervals postinfection, AAF and CAM of embryos were harvested. AAF was assayed for AAAV and CELO virus CF antigens, and thin sections of the CAM were examined for virus particles with the electron microscope. Symbols: (+) indicates the quantity of virus seen at various times postinfection, (ND) not done.*

was studied by antibody-mediated aggregation of virions. The reaction was visualized by electron microscopy (Fig. 6). The aggregates appeared to be composed of groups of virus particles cross-linked by fibrous strands which were interpreted to be antibody molecules. AAAV was specifically aggregated by homologous antiserum, but not by antiserum specific for AAV types 1, 2, 3, and 4 (Table 4). Similarly, AAV types 1, 2, 3, and 4 were specifically aggregated by homologous sera.

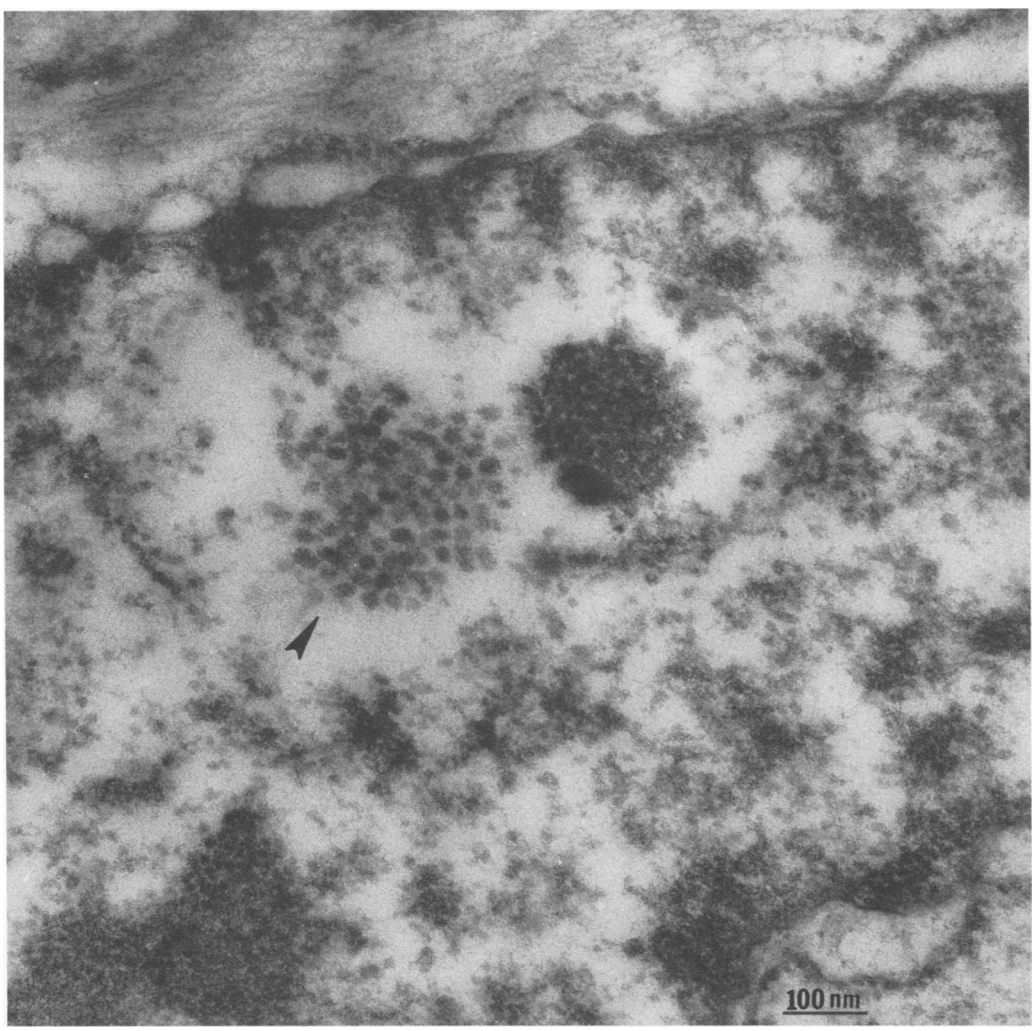


FIG. 5. *Thin section of a chicken embryo CAM cell. Arrow indicates a cluster of AAAV particles within the nucleus. Embryo was coinfected with AAAV and CELO virus. The membrane was harvested 48 h postinfection.*

TABLE 3. *Induction of AAAV and CELO virus CF antigens in chicken embryo kidney cells*

| Time (h) | IF antigen | | CF antigen[a] | |
|---|---|---|---|---|
| | CELO | AAAV | CELO | AAAV |
| 0 | 0 | 0 | <2 | <2 |
| 10 | 0 | 0 | <2 | <2 |
| 20 | 0 | 0 | <2 | <2 |
| 24 | +(<1)[b] | +(<1) | <2 | <2 |
| 48 | +(75-85) | +(50-60) | 4 | <2 |
| 96 | ND[c] | ND | 8 | 4 |

[a] In supernatant fluids.
[b] Numbers in parentheses represent percentage of cells showing IF antigen.
[c] ND = not done.

TABLE 4. *Aggregation of avian and primate AAV by homologous and heterologous antisera*

| Antiserum | Virus | | | | |
|---|---|---|---|---|---|
| | AAAV | AAV-1 | AAV-2 | AAV-3 | AAV-4 |
| AAAV | +[a] | – | – | – | – |
| AAV-1 | – | + | – | – | – |
| AAV-2 and 3 | – | – | + | + | – |
| AAV-4 | – | – | – | – | + |
| Normal | – | – | – | – | – |

[a] Symbols: (+) aggregation of virus particles, (–) no aggregation.

## DISCUSSION

Dutta and Pomeroy (5) observed 20-nm particles in a preparation of quail bronchitis virus which they interpreted as internal components of mature adenovirions. Later it became apparent that these particles were morphologically similar to the adeno-associated viruses (9). Recently, McFerran et al. observed 20-nm particles associated with adeno-viruses they isolated from chickens in Ireland (15). The small virus characterized in this report was found in a suspension of the Olson isolate of quail bronchitis virus (20).

After density gradient centrifugation, peaks of antigenic material appeared at densities of 1.39 and 1.42 g/cm$^3$. The bulk of the CF antigenic material appeared at the former density; the infectious particles appeared at the latter. These densities correspond to those reported for the parvoviruses (10). By electron microscopy, the 1.39 and 1.42 g/cm$^3$ bands appeared to represent incomplete and complete forms of the virus, respectively.

Measurements of particle diameter ranged from 18 to 21 nm, a result consistent with those of strains of other AAV (18, 23). Although the virions were hexagonal in outline, a definite pattern of symmetry could not be resolved. No variation in size was detected in virus particles banding at the different densities.

As reported for AAV (18), the avian AAV contained single-stranded DNA when tested by acridine orange staining. The virus was stable in heat and chloroform, and in this respect it also resembles AAV from other sources (11, 23). It was not affected by low pH and did not agglutinate erythrocytes at either 4 or 37 C. Both AAV type 4 (9) and bovine AAV X7 agglutinated red blood cells (13).

Microagglutination was used to differentiate AAAV from AAV types 1 to 4. This method adequately distinguished the avian virus from the morphologically similar primate viruses. The fibrous strands connecting the aggregated particles were similar to antibody molecules described by Almeida et al. in negatively stained preparations of antigen-antibody complexes (1).

A distinguishing characteristic of the adeno-associated viruses, their defectiveness, was demonstrated in studies of the replication of AAAV in chicken embryos in vivo and in CEK cells in vitro. Replication of the virus was detected by immunologic methods or by electron microscopy only in embryos or cultures of cells coinfected with adenovirus. In ultrathin sections of infected embryonic membranes, helper adenoviruses were detected at an earlier time than was the AAAV. The two viruses were not readily found in the same cell, demonstrating the "mutual interference" described by Mayor et al. (17) and others (11, 21).

The characteristics of avian AAV and its mode of replication are essentially the same as those described for adeno-associated viruses of other species. This serologically distinct virus should be considered a candidate for the defective group of parvoviruses.

FIG. 6. *(A) Negative stain (1% phosphotungstic acid) of AAAV after incubation at 37 C with rabbit serum directed against a heterologous AAV. (B) Negative stain (1% phosphotungstic acid) of AAV after incubation with homologous serum. Arrow points to fibrous strands of antibody molecules.*

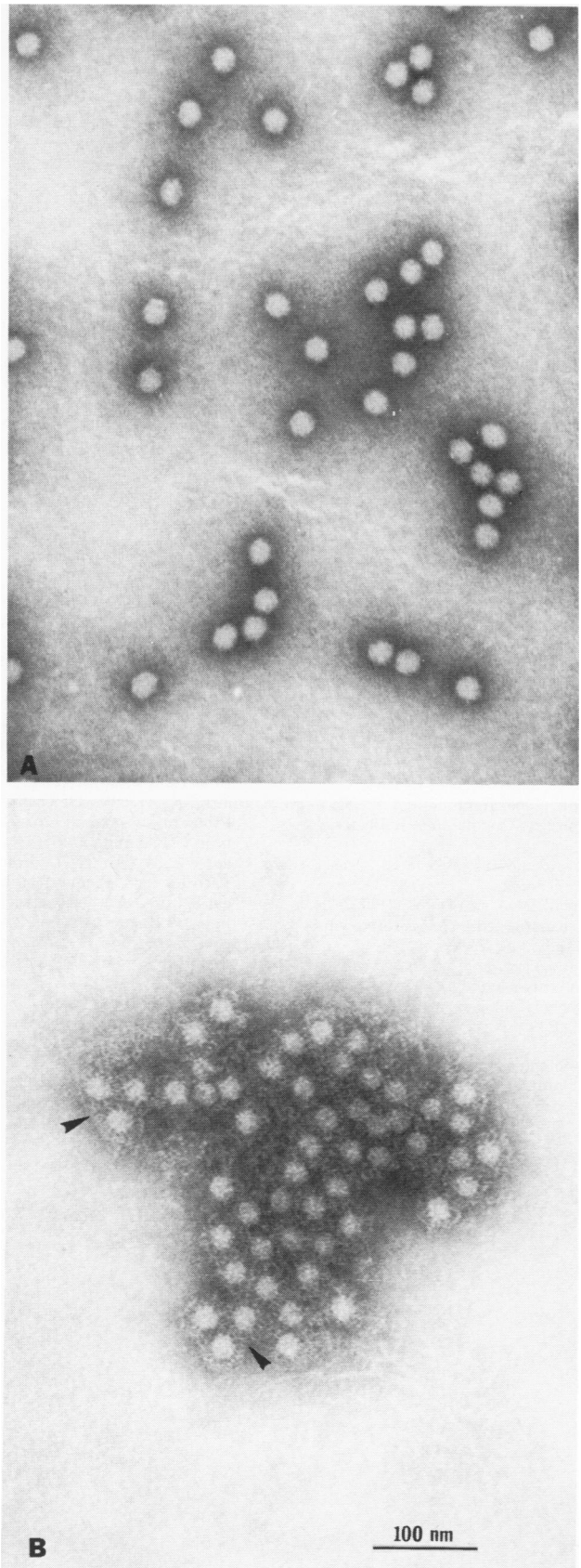


FIG. 6 A and B

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